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Changes in lactate dehydrogenase isozyme pattern during the development of *Xenopus laevis* (Daudin)

by

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with 8 figures

Lactate Dehydrogenase (LDH) of mammalian tissues occurs in five molecular forms (isozymes), which are composed of two subunits, A and B, synthesized at separate gene loci. They randomly assemble into all possible combinations of four (A_4, A_3B_1, A_2B_2, A_1B_3, B_4), which are readily separated by electrophoresis. The LDH isozyme patterns are species and tissue specific. They undergo changes during development (Cahn, *et al.*, 1962; Markert, 1963; Shaw and Barto, 1963). A third subunit, C, has been found in the testis of several mammals and birds (Blanco and Zinkham, 1963; Goldberg, 1963; Zinkham, *et al.*, 1969).

Various species of the amphibia have been studied for LDH pattern, and the number of isozymes found varies from 2 to 25 (Chen, 1968; Goldberg and Wuntch, 1967; Grainger and Kunz, 1966; Kunz and Hearn, 1967; Moyer *et al.*, 1968; Nace *et al.*, 1961). Therefore, the amphibian isozymes do not fit in with the hypothesis of random aggregation of two subunits put forward for mammalian tissues.

Xenopus laevis was shown to have nine isozymes in its adult organs (liver excepted). When subjected to electrophoresis the patterns are tissue specific; heart and muscle show highest activity at opposite ends of the banding pattern. During early ontogeny, i.e. from fertilization to early tailbud stage, four anodic isozymes are resolved. Subsequently, the most cathodic isozyme appears and the full complement of nine isozymes is achieved at the hindlimb bud stage (Kunz and Hearn, 1967). Similar results are reported by Glaycomb and Villee, 1971).

The purpose of this research was to study the LDH isozymes of 1. isolated regions of egg, cleavage and gastrula stages, 2. isolated tissue primordia of neurula

and tailbud stages, 3. organs such as heart, muscle, brain, eye, intestine from the embryo to the adult toad. The changes in pattern observed are compared with histological data.

MATERIALS AND METHODS

Material: Adult *Xenopus laevis* (above 7 years of age) from laboratory stock was used. To obtain spawn, 400 I.U. of the choriogonadotrophic hormone Pregnyl (Organon) were injected into males and females. The spawn were raised at 22°C, and were fed on nettle powder *(herba urticae)* during the larval period and on minced ox liver after metamorphosis. Developmental stages to the end of metamorphosis were determined according to NIEUWKOOP and FABER (1967) (table 1).

After metamorphosis the young toads were tested at 1, 2 and 3 weeks. From then onwards, they were aged according to weight, until sexual maturity was reached (males at 12 months and of approximate weight 15 g; females at 16 months and of approximate weight 25 g).

Agar gel electrophoresis: The LDH isozymes were separated electrophoretically on agar gel by the high voltage plate method of WIEME (1965). The electrophoresis tank, maintained at constant temperature, was by Vitatron. The agar gel was supported by microscope slides (75 × 25 mm). Three slides with 2-3 samples each were tested in each run. Electrophoresis was carried out for eighty minutes at a voltage gradient of 25 V/cm and at 12°C. LDH activity was visualized by incubating the slides for 45 min. at 37°C in the dark in an agar solution containing 204.5 mg of DPN (Diphosphopyridine nucleotide), 54.5 mg of INT (Iodonitrotetrazolium), 5.5 mg of PMS (Phenazine methosulphate), and 10.2 ml of neutralized lactic acid, with bidistilled water added to make a final volume of 100 ml (WIEME, 1965).

Preparation and application of samples: a. Direct tissue method: Different regions of early developmental stages were isolated with a microscalpel, after placing the material on a chuck precooled at —20°C in a kryostat (fig. 1). From the tailbud stage 25 onwards the specimens were microdissected, using watchmaker's forceps, under a Wild Stereomicroscope M 5. The fragments or organs thus obtained were pushed into an incision made in the agar gel. In order to avoid desiccation, dissection and application to the gel have to be immediate; no weighing is possible. b. Organs from young and adult toads were applied as homogenates. The organs were ground in a micro glass-homogenizer (Vel), or directly in microcentrifuge tubes of 2 ml vol., at low temperature. Centrifugation was performed in a microcentrifuge (Ole Dich, Hvidovre, Denmark) for 20 min. at 17 000 r.p.m. in a coldroom kept at 4°C. 6 λ of supernatant were applied to each slit.

Histological data : The comprehensive histological observations contained in the Normal Table of *Xenopus laevis* (NIEUWKOOP and FABER, 1967) were used. Additional data were obtained from histological treatment of own material:

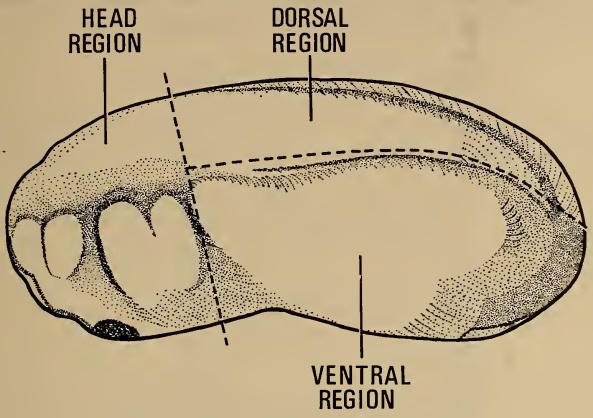

FIG. 1.

Embryo of stage 24 (early tailbud).
The broken lines indicate how the embryo was subdivided. Length 2.5 mm.

Specimens at stages 23-49 were preserved in Bouin (Duboscq), sectioned at 7 μ and stained with acid Hemalum (Maier)/Orange G and Azan (after Heidenhain).

RESULTS

The bands of the electrophoretic patterns are numbered I-IX, 1 denoting the most anodic and IX the most cathodic band.

1. LDH PATTERN OF DIFFERENT REGIONS OF EARLY EMBRYONIC STAGES.

Fertilized egg to early tailbud stage (stages 1-24) display four anodic bands (I-IV) when analyzed *in toto* (KUNZ and HEARN, 1967). Electrophoresis of fragments of these stages give the following results: Analyses of animal and vegetal halves, as well as of dorsal and ventral halves, of cleavage stages 1-9 show four isozymes as in the whole egg. Also, their relative intensity is the same, with band I the strongest, followed in intensity by bands II, IV and III. Fractions of gastrulae, neurulae, to early tailbud (stages 10-24) again do not show any difference between

one another, either in regard to the number or to the relative intensity of the bands (figs. 2, 5).

At the tailbud stage 25, which is characterized by the appearance of the most cathodic isozyme (IX) in whole specimens (KUNZ and HEARN, 1967), band IX is shown to be restricted to the dorsal and head regions (fig. 2).

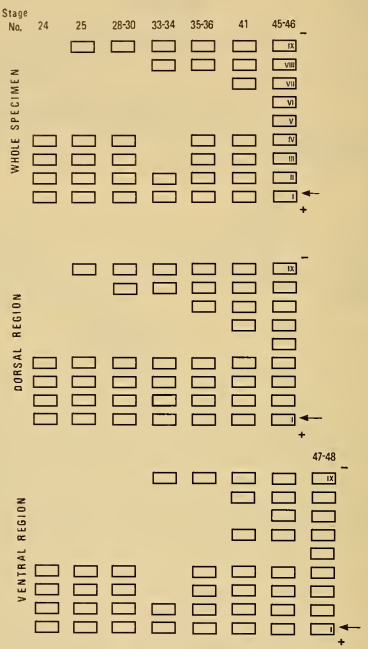

FIG. 2.

Diagrammatic representation of LDH isozyme patterns of whole specimens compared with isolated dorsal and ventral regions.

ad figs. 2-8.

← point of application of sample.
(h) homogenate. All other samples applied as "direct tissue".

Between the tailbud and hindlimb bud stages (stages 25-46) the remaining bands appear to make a total of nine in whole animal homogenates (KUNZ and HEARN, 1967). Analyses of different parts of embryos and larvae give the following results: At stage 28 the tailbud is sufficiently elongated to be inserted into the gel, so that from now onwards, the dorsal region may be subdivided into trunk and tail and run against the ventral region. The patterns and their development are

given (figs. 2 and 3). The trunk pattern is initially ahead of the tail pattern, but at stage 41 both have attained the full complement of nine isozymes. The ventral region, however, still displays only four bands (I-IV) at stage 28. Between stages 32 and 34 bands III and IV are temporarily lost; at stage 34 band IX appears and the set of nine isozymes is attained at stage 47/48.

2. The development of organ patterns.

Trunk muscle: At stage 41 the trunk region displays nine bands (fig. 3). In a series of experiments the trunk muscles were cleared from neighbouring tissues.

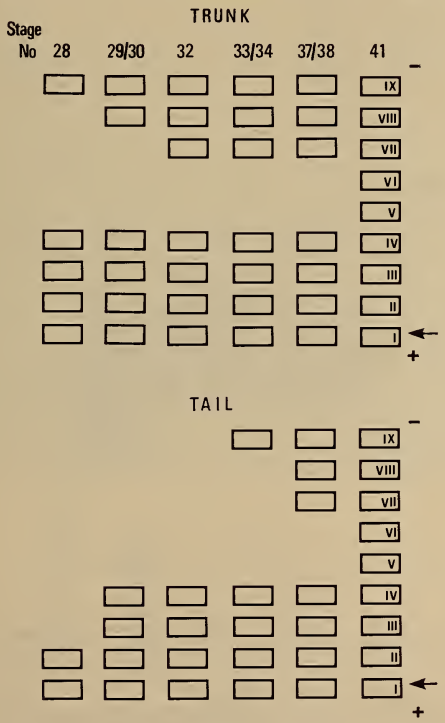

Fig. 3.
LDH isozyme pattern of trunk and tail, obtained by bisecting the dorsal region (fig. 2).

The analyses of muscle tissue alone show the same pattern as the whole trunk. Although the full number of isozymes is present at this stage, their relative inten-

sities differ from those of the adult skeletal muscle: as in the adult, bands VII-IX are most prominent at stage 41; bands V and VI, however, are only weakly staining and band I stains intensely. From stage 41 onwards band I gets progressively weaker, and from stage 49 onwards bands V and VI increase in intensity, so that at stage 51 distribution of activity as in the adult muscle (m. longissimus dorsi) is attained.

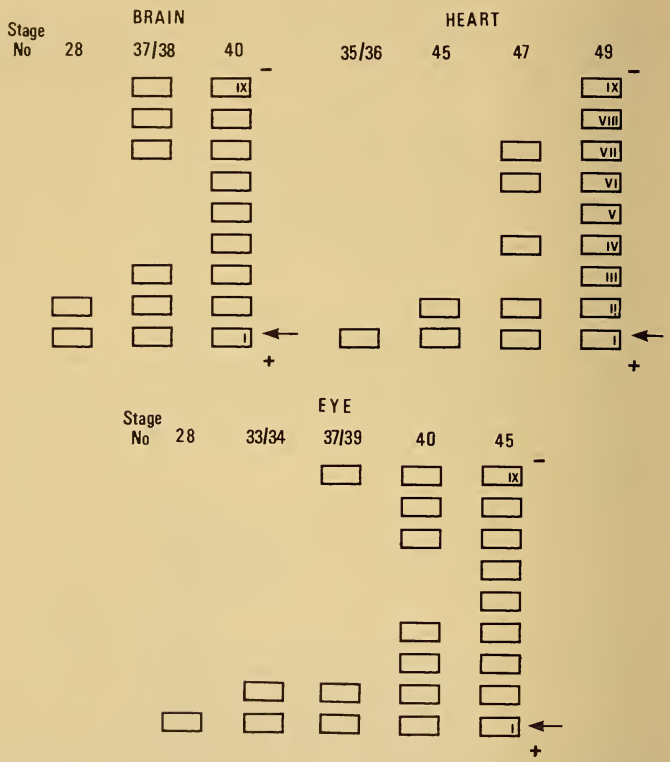

Fig. 4.

Ontogenetic changes of LDH pattern of single organs.

Tail muscle: At stage 41 nine isozymes are established in the tail (fig. 3). Here again, experiments with isolated muscle give the same LDH pattern as for the whole tail, in regard to both number and relative intensities of bands. The

relative intensities of different bands at stage 41 are, however, different from adult skeletal muscle. They are similar to that of trunk muscle at the same stage (41). At stage 51, the tail muscle shows the same distribution of activity as adult skeletal muscle. (The patterns of all adult skeletal muscles tested—individual trunk, hindlimb and forelimb muscles—are identical). The pattern of activity persists to stage 64/65 when band IX decreases abruptly in intensity (fig. 6).

Heart: At stage 35/36 isozyme I is resolved, followed by isozyme II at stage 45. The next change is observed at stage 47, when a strong band IV and weak bands VI and VII are resolved. The ninebanded pattern is established at

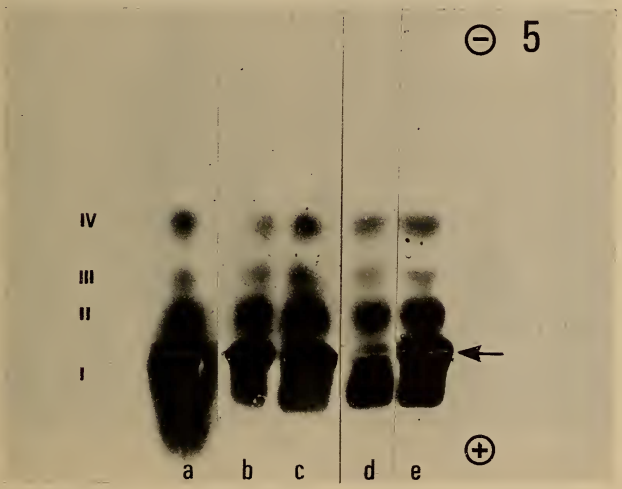

FIG. 5.

Electrophoretic pattern of LDH isozymes of stage 5.
a. one whole egg b. animal half c. vegetal half
d. dorsal half e. ventral half.

stage 49; it shows the characteristic distribution of the adult heart, in that isozymes I-IV are most prominent (figs. 4, 6). The pattern does not change during later larval, metamorphic, postmetamorphic and young toad stages.

Eye: A weak band I becomes apparent at stage 28. Isozyme II is first detected at stage 33/34. At stage 37/38 isozyme IX becomes resolved, followed by III, IV, VII and VIII at stage 40. By stage 45 all bands are present and show activities similar to the adult eye (figs. 4, 7). This pattern does not change during the ensueing stages to adulthood.

Brain: At stage 28, the brain contains isozymes I and II, with isozyme I the more prominent. A change occurs at stage 38 when an intensely staining band IX and weakly staining bands III, VII and VIII appear. At stage 40 all nine isozymes are present. Their relative intensities, however, differ from those of the adult

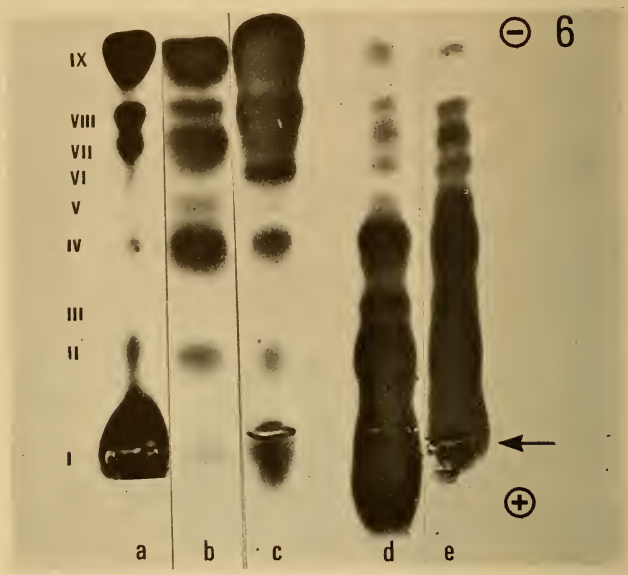

FIG. 6.

Electrophoretic pattern of LDH isozymes of tail muscle and heart.
a. tail stage 41 b. tail stage 51 c. skeletal muscle adult (h)
d. heart adult (h) e. heart stage 49.

brain, in that band VII is stronger than band VIII and band VI is very weak. The changeover to adult pattern takes place at stage 47 (figs. 4, 7). The pattern remains the same throughout larval, metamorphic, postmetamorphic and young toad stages. It should be mentioned, however, that band IX of the larval brain is labile. In a few experiments with different stages it showed up only faintly.

Intestine: The ventral region of stage 47/48 reveals nine isozymes (fig. 2). At this stage, the yolk is absorbed and the intestine coiled with two spirals. Analysis of the intestine gives the same pattern as the ventral region. Detailed analysis of the developing LDH pattern of the alimentary canal was not carried

out during this study. Spot checks of whole intestines were, however, made, and it becomes evident that from stage 51 to 62 the cathodic isozymes (V-IX) are most prominent, whereas at stage 63/64 a shift of activity to the anodic isozymes is observed. The pattern of the adult duodenum shows, again, greatest activity in the cathodic isozymes.

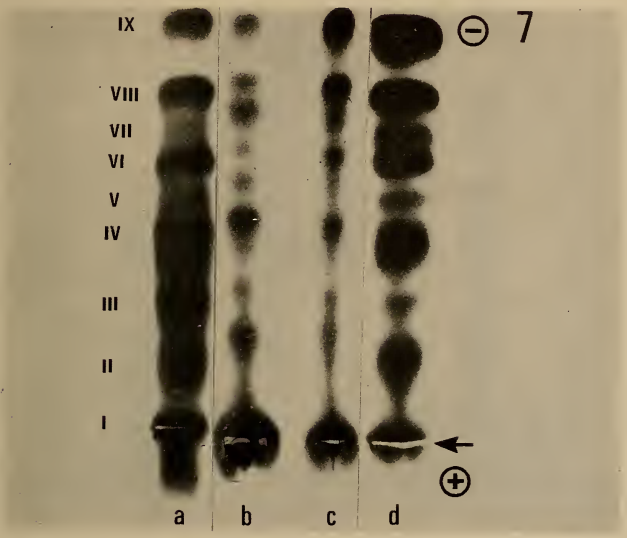

FIG. 7.

LDH isozymes of brain and eye.
a. brain adult (h) b. brain stage 40 c. eye stage 45 d. eye adult (h)

Hindlimb muscle: At stage 54 (paddle stage) the limb bud is elongated enough to be dissected and inserted into the gel. All the nine isozymes are resolved. There is no predominant cathodic activity as in the adult skeletal muscle. From stage 61/62 onwards band 1 stains up only weakly, and at one week after metamorphosis bands II and III, too, are very low in activity. Thus, the distribution of activity now resembles adult leg muscle except that isozyme VII stains up more intensely than VI. Toads from 0.6 g weight onwards show activity of band VI higher than VII (fig. 8).

Forelimb muscle: At stage 58 the forelimb bud can be tested. All nine isozymes are established showing isozymes I-IV as the most prominent. At stage 63/64 a

shift in activity towards the cathodic isozymes is observed. The pattern is similar to that of the forelimb muscle of an adult toad, except that band VII is more intense than band VI. Finally, in toads from 1.0 g onwards isozyme VI is more active than VII. Parallel runs, on the same slide, of forelimb and hindlimb muscle homogenates show the anodic isozymes of the former much more active (fig. 8).

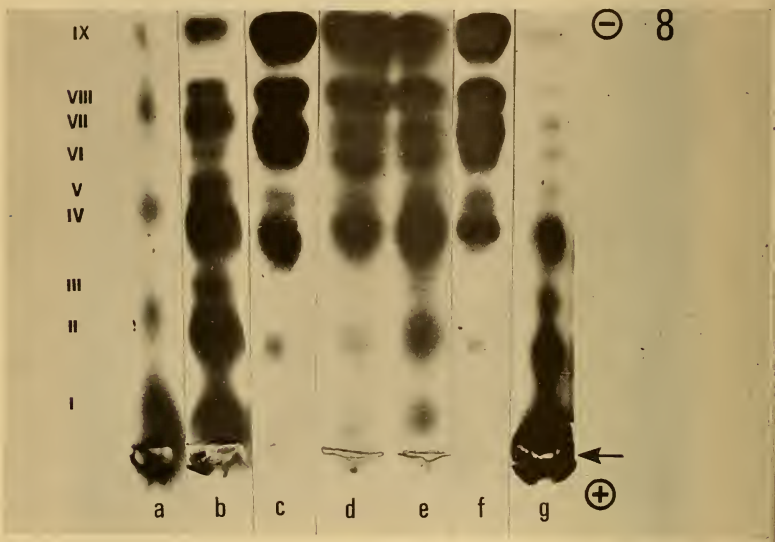

FIG. 8.

LDH isozymes of hindlimb and forelimb muscle.
a. hindlimb stage 54 b. hindlimb stage 61/62
c. hindlimb 1 week after metamorphosis (h) d. hindlimb adult (h)
e. forelimb adult (h) f. forelimb 1 week after metamorphosis (h)
g. forelimb stage 58.

DISCUSSION

The "direct tissue method" proves to be a particularly suitable method for embryological electrophoretic work. It allows, for instance, the analysis of fractions of single amphibian eggs of 1.5 mm diameter and minute brains and eyes taken from embryos of 4 mm length. New isozymes are detected much earlier during development than when homogenates are applied, because no addition of buffer,

which dilutes enzyme concentration, is required. One of the drawbacks of this method is that when organs or tissue fragments are inserted into the gel, they often distend the application slit, which results in irregular bands ("bat-wings"). Also, since the weight of the tissue fragments or organs is not known, only within each sample may the activities of the isozymes be compared.

The LDH pattern from newly fertilized egg to early tailbud (stages 1-24) is the same for the different regions. A change is observed at stage 25, when isozyme IX appears and proves to be restricted to the dorsal and head regions. The appearance of this isozyme, which is the most prominent in adult skeletal muscle, may be correlated with the onset of contractility (stage 23), which in embryonic vertebrate muscle generally occurs at the time, or shortly before, the first myofibrillae arise. (GLAYCOMB and VILLEE, 1971, observed the first appearance of band IX only at stage 27, which may be due to the fact that they analyzed whole embryos). Also in the other regions, isozyme IX appears at similar stages of muscle cell development: In the ventral region, it is resolved for the first time at stage 33/34, when the muscle cells which are destined to form the ventral somatic muscles ("Urwirbelfortsatz" of MAURER (1891)) have started to migrate to this region. The cells form first a double layer, which, by desintegration of the lateral row of cells, is then reduced to a single row. At the same time the "Urwirbelfortsatz" detaches itself from the dorsal somatic musculature, a process which is completed at stage 41 according to RYKE (1953), and at stage 38 according to NIEUWKOOP and FABER (1967). Myofibrillae appear at stage 38/39 (own observation). The cells are transformed into muscle fibres only at stage 44 (NIEUWKOOP and FABER, 1956). In the tail, isozyme IX appears for the first time at stage 33/34, shortly before the myofibrillae are formed in the most anterior tail segments (table 2). It disappears, on the other hand, at stage 64, when the actual degeneration of the tail muscle has started (49 postotic segments at stage 63 are reduced to 14 at stage 64).

All embryonic organs and regions tested display first the anodic isozymes. The ninebanded pattern in heart, brain, eye, trunk muscle, tail muscle and intestine is achieved, in each case, once the intracellular yolk platelets have disappeared. This seems to suggest that the presence of yolk may have an inhibitory effect on the expression of some LDH isozymes. Adult distribution of activity within the ninebanded pattern, however, is not attained at comparable histological stages: In the eye, the first appearance of the adult pattern (stage 45) coincides with the establishment of the rods and cones. Their appearance and relative numbers at this stage are the same as in the adult, although they are not yet fully grown. The brain has all its parts well developed at stage 53, so that later development consists mainly of growth and some further cytological differentiation. The adult LDH pattern, however, is seen already at stage 47, at a time when the cerebral hemispheres are developing and the vascularization of the brain has started. The heart

displays adult pattern at stage 49, when the atrial septum has been established and the trabeculae are in the process of formation. In the hindlimb muscle a shift towards adult expression (i.e. lower intensity of the anodic bands) is observed at stage 61/62; this is one stage after histogenesis is completed and some time after spontaneous movements of the legs have started (stage 58). In the forelimb muscle the same change is observed at stage 63/64, long after histogenesis is completed (stage 58) and before the forelimbs are used for food catching (from stage 66 onwards). Adult expression of the whole pattern is achieved, in the muscles of both extremities, only several weeks after completion of metamorphosis.

The technical assistance of James Dunne, Matthew Foster and Margaret McNair, all of this department, is gratefully acknowledged.

Summary

The electrophoretic patterns of Lactate Dehydrogenase Isozymes in the developing *Xenopus laevis* are examined, and the observed changes are compared with histological data.

Four isozymes are present in the fertilized egg, whereas in all adult organs (liver excepted) nine isozymes are found. The fourbanded pattern persists during early development until the tailbud stage. Isolated parts give the same result as whole embryos. Regional differences, however, are observed from the tailbud stage onwards, when the most cathodic isozyme is resolved. It makes its first appearance in the dorsal region and much later in the ventral region and tail. In each region it appears at a time when myofibrillae have begun to form.

Extirpated organs—heart, brain, eye and gut—are examined from their anlage and followed through embryonic, larval, metamorphic and postmetamorphic stages until sexual maturity of the animal is reached. In all organs the anodic isozymes develop first, and the adult number of nine bands is achieved in each, once the intracellular yolk platelets have disappeared. Similarly, in trunk and tail muscle nine isozymes are resolved once the yolky material is consumed. Adult expression of the ninebanded pattern, however, does not seem to be associated with comparable histological events; it is achieved at widely different developmental stages of the organs and tissues tested.

Résumé

L'auteur a étudié l'évolution des formes multimoléculaires de la déshydrogénase lactique (LDH) pendant l'ontogénèse de *Xenopus laevis*. Les observations sont mises en rapport avec des données histologiques.

L'électrophorèse en gel d'agar révèle neuf formes de lacticodéshydrogénases dans les organes du *Xenopus* adulte (sauf le foie), tandis que l'œuf fécondé possède quatre formes. Dans les jeunes stades de développement — segmentation, gastrulation, neurulation — le « pattern » ne change pas. De plus, les régions isolées donnent le même résultat que l'embryon intact. Cependant, des différences régionales sont observées dès le stade du bourgeon caudal, lorsque l'isozyme migrant le plus rapidement vers le pôle négatif apparaît. Cette isozyme est observée d'abord dans la région dorsale de l'embryon; beaucoup plus tard on la trouve dans la région ventrale et dans la queue bourgeonnante. L'apparition de cette isozyme coïncide, dans toutes les régions, avec la différenciation des myofibrilles.

Plusieurs organes et tissus — le cœur, le cerveau, l'œil, l'intestin, la musculature striée — ont été analysés à partir de leur primordium. Ce sont toujours les isozymes anodiques qui apparaissent les premières. Le nombre adulte (9) est réalisé, dans tous les organes, après la disparition des plaquettes vitellines; mais les « pattern » spécifiques d'organe sont atteints à différents stades de développement des organes.

Zusammenfassung

Das Laktatdehydrogenase-Muster des sich entwickelnden *Xenopus laevis* wird mittels Agargel-Elektrophorese untersucht. Die beobachteten Veränderungen des Musters werden mit histologischen Daten in Beziehung gebracht.

Das befruchtete Ei enthält vier Isozyme, während die adulten Organe deren neun aufweisen. Während der jungen Entwicklungsstadien (Furchung — frühes Schwanzknospen-Stadium) verändert sich das 4-Banden-Muster nicht. Isolierte Teile ergeben dieselben Resultate wie der Totalkeim. Regionale Verschiedenheiten des Musters werden jedoch vom Schwanzknospen-Stadium an beobachtet. Das am schnellsten wandernde kathodische Isozym erscheint zuerst in der dorsalen Region. Erst viel später wird es in der ventralen Region und dem auswachsenden Schwanz nachgewiesen. In allen Regionen fällt das erste Auftreten dieses Isozyms mit dem Beginn der Myofibrillen-Bildung zusammen.

Verschiedene Organe — Herz, Gehirn, Auge, Darm, Muskulatur — werden separat untersucht. Die Entwicklung ihres Musters wird von der Anlage über Embryonal-, Postembryonal-, Metamorphose-, Postmetamorphose-Periode bis zum geschlechtsreifen Tier verfolgt. In allen Organen erscheinen die anodischen Isozyme zuerst. Die adulte Bandenzahl (9) wird in jedem Falle erreicht, wenn die intrazellulären Dotterplättchen resorbiert sind. Das adulte Verteilungsmuster hingegen tritt in den verschiedenen Organen zu verschiedenen Zeitpunkten ihrer Entwicklung auf.

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Table 1

Short description of developmental stages of Xenopus laevis

(based on the Normal Table by Nieuwkoop and Faber, 1967)

Stage No.	
1- 9	Cleavage.
10-12	Gastrulation.
13-20	Neurulation.
21	Primary eye vesicles.
24	Tailbud discernible. Initial motor reactions to external stimulation.
25	Beginning of tailfin formation.
26	Ear vesicles protruding. Myotomes showing through for the first time. Beginning of spontaneous movements.
28	Fin extending to anus.
29/30	Eye cup grey.
32	Eye cup horseshoe shaped.
33/34	Beginning of heart beat.
35/36	Eye entirely black. Beginning of hatching. Length of tailbud about 3 × its breadth.
40	Mouth breaks through. Beginning of blood circulation in gills.
41	Gut starts coiling.
44	Appearance of tentacle rudiments. Blood circulation in gills ceased.
45	Beginning of feeding.
46	Hindlimb bud visible.
48	Forelimb bud visible.
53	Hind- and forelimbs in paddle stage.
58	Beginning of metamorphosis: Forelimbs in process of eruption. Ultimate length of larva.
59	Tentacles begin to shrivel up.
61	Head narrower. Tentacles considerably shortened, curved backwards. Fins considerably reduced.
63	Head harrower than trunk. Tentacles mostly disappeared. Tail still longer than body.
64	Length of tail 1/3 body length.
66	Tail only small triangle. End of metamorphosis.

Table 2

Development of different organs.

(based on the Normal Table by Nieuwkoop and Faber (1967).
* additional own observations)

	Trunk muscle
20	Myoblasts spindleshaped in most anterior somites.
21	Myofibrillae formed in most anterior somites.
29/30	Segregation of myotomes reaches tail region.
33/34	Cross-striations of myofibrillae.*
41	Yolk consumed.
	Tail muscle
32	Myoblasts spindleshaped in anterior tail somites.
35/36	Myofibrillae visible in anterior tail somites.
39	Cross-striations of myofibrillae.

Stage No.	
40	Yolk platelets rapidly disappearing.
42	Yolk consumed.
43	Histogenesis of myotomes completed.
44-58	Growth in size of muscle segments.
	Hindlimb muscle
52	Condensation of mesenchyme.
53	Histogenesis of muscle fibres started.
55	All major muscles of limb present; most have established their origin.
60	Full differentiation of muscles. Differ from adult only in relative small amount of connective tissue.
	Forelimb muscle
55	Beginning of muscle differentiation.
56	Myoblast cells formed.
58	All muscles well developed.
	Brain
28	General pattern mainly established.
28-40	Fibre tracts and later commissures develop. Yolk gradually consumed.
40-41	Yolk used up.
43-46	Major development of cerebral hemispheres. Penetration of blood vessels into brain.
53	All parts well developed. Later development mainly growth and some further cytological differentiations.
	Heart
28	Primordium of endocardial tube appears.
33/34	Myocardial wall completed.
35/36	S-Shape; chambers distinct. Red blood cells present.
41	Thickening of myocardium of conus and ventricle. Trabeculae develop in ventricle.
44/45	Atrium partitioned. All valves developed.
48	Yolk consumed.*
	Eye
25	Primary eye vesicles fully developed.
27-31	Invagination of eye vesicles.
33/34	Lens primordium detached from ectoderm.
35/36	Beginning of differentiation of pars optica retinae.
37-41	Vascularization of retina by arteria hyaloidea.
39	Primary morphogenesis of eye completed.
40	Nuclei of central lens fibres degenerating.
42	Rods and cones distinguishable.
39-44	Development of eye muscles and attachment to sclera.
45	Yolk consumed.
47/48	Appearance of rods and cones, and their relative number, same as in adult, although not yet fully grown.
49-66	Final growth of rods and cones.